

Analysis of the complexity of the multimeric structure of factor VIII related antigen/von Willebrand protein using a modified electrophoretic technique

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SUMMARY A method for multimeric analysis of factor VIII related antigen/von Willebrand protein is described. By modifying an existing method the technique has been simplified and optimised so that the different molecular forms of factor VIII related antigen and their triplet structure can be visualised. Clear differences can be seen between patterns in normals and type II_A von Willebrand's disease variants in that the latter lack high and intermediate multimers and also have a different configuration to the triplet pattern in the lower molecular weight multimers. Differences in triplet pattern can also be seen in different type II_A patients. The advantages and further application of this method for investigating the molecular defect in von Willebrand's disease are discussed.

A high molecular weight protein, factor VIII related antigen/von Willebrand protein (VIII:AG) is associated with factor VIII procoagulant protein (VIII:C).¹⁻⁴ The structural complexity of VIII:AG has been studied with electrophoresis using large pore polyacrylamide,^{4,5} or agarose and acrylamide gels.⁶⁻⁹ Early studies suggested that normal VIII:AG consists of an aggregating series of polydispersed oligomers.⁴ Similar studies of this protein in patients with von Willebrand's disease have been done and classifications proposed.¹⁰⁻¹² Electrophoresis of VIII:AG on a sodium dodecyl sulphate (SDS) glyoxyl agarose gel¹³ and a commercially available rehydratable SDS agarose gel¹⁴ have allowed the identification of high, medium and low molecular weight oligomers. Ruggeri and Zimmerman have referred to these analyses as multimeric and have proposed a classification of subtypes of von Willebrand's disease.¹⁵ They subsequently reported another method of SDS gel electrophoresis using a discontinuous buffer system providing increased resolution.¹⁶ We report further modification of this technique which provides even more precise resolution of the VIII:AG multimeric bands.

Material and methods

REAGENTS

Unless otherwise stated these were as described by Ruggeri and Zimmerman,¹⁶ and the chemical reagents

used were supplied by either BDH Chemicals Ltd or Sigma Chemical Company.

Rabbit anti-human factor VIII immunoglobulin (IgG fraction) was supplied by DAKO Immunoglobulins a/s, Mercia-Brocades Ltd. and after labelling with ¹²⁵I from Radiochemicals Centre, Amersham, used for the final autoradiography.

IgM

IgM purified from a patient with IgM myeloma was used as a molecular marker of known molecular weight.

PATIENTS AND CONTROLS

Venous blood was obtained by venepuncture from normal adult men and selected severe type I and type II_A von Willebrand's disease patients. Nine parts of blood were added to one part of 0.11 molar trisodium citrate. Platelet poor plasma was prepared by centrifugation at 1500 g for 15 min at 4°C. Separated plasma was snap frozen at -20°C and thawed at 37°C before use. Patients studied were LW type I or severe classical vWD (VIII:C, VIII:AG and VIII:Wf all zero); HW type II_A or variant vWD (VIII:C 0.4 U/ml, VIII:AG 0.63 U/ml and VIII:Wf 0.05 U/ml.) and CB (VIII:C 0.54 U/ml, VIII:AG 0.86 U/ml and VIII:Wf 0.05 U/ml.).

SDS GEL ELECTROPHORESIS

The method of VIII:AG multimeric analysis

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described by Ruggeri and Zimmerman¹⁶ has been modified by altering the electrophoretic conditions (current and time of electrophoresis), by increasing the gel size and by using a labelled commercial anti-human VIII:AG antibody (IgG) instead of an affinity purified antibody for the autoradiography.

Gel preparation

Agarose gels (1.4% and 2%) and an agarose (0.8%) and acrylamide (2.5% with 5% cross linking) gel were used. A large gel to take 13 samples was prepared by pouring 25 ml of hot unpolymerised running gel in a sandwich set (17 × 12 × 0.1 cm) made of a piece of gel bond film covering one glass plate and separated from a second glass plate by a 1 mm thick U-shaped plastic spacer. The two glass plates are held together with binder clips. After the running gel has set, the top glass plate is removed and a 3–5 mm strip from the top of the gel is cut away and discarded. The top glass plate is resealed into its original position and 5 ml of stacking gel poured above the running gel and left to set for one hour at 4°C. Thirteen rectangular sample wells (8 × 2 mm) were cut in the stacking gel at 5 mm intervals and 10 mm from the interface with the running gel.

Electrophoretic conditions

Different conditions were tested (see Results) in a Pharmacia FEB-3000 flat bed electrophoretic tank. Optimal electrophoresis was achieved with a constant initial current of 1.9 mA/cm from a Pharmacia ECP 3000/150 power supply until the samples had migrated from the wells. The empty wells were filled with stacking gel and electrophoresis. A Pharmacia volt-hour integrator (mA/cm). Electrophoresis is stopped when the tracking dye has moved 10 cm from the wells. This is usually achieved after 8 h. However, if more convenient, similar results could be obtained if a current of 1.2 mA/cm is used during overnight electrophoresis. A Pharmacia volt-hour integrator (VH-1) coupled to the power supply registered 2000–2500 volthours for each run. After electrophoresis the gels are fixed washed and dried as described by Ruggeri and Zimmerman¹⁶ prior to autoradiography.

In the thirteenth well purified IgM is run as a molecular weight marker. This strip of dried gel is cut away and stained with Coomassie brilliant blue.

AUTORADIOGRAPHY

The dried plates are incubated overnight in a commercial rabbit anti-human VIII:AG immunoglobulin labelled with ¹²⁵I by the Chlorine gas method¹⁷ at specific activity of 10–20 mCi per mg protein and diluted in 60 ml of veronal buffer to 1 × 10⁶ cpm/ml. After incubation the gels are washed thoroughly in

saline followed by distilled water and then dried. Autoradiograph plates are produced using a Dupont Cronex 4 film in a cassette fitted with Dupont Quanta II intensifying screen. The plates have to be kept at –70°C for 2–3 days to allow clear definition of the bands.

Results

Clear differences could be observed (Fig. 1) between the multimeric patterns of normal plasma and that from a patient with type II_A von Willebrand's disease obtained after electrophoresis on 1.4% and 2% agarose gels and 0.8% agarose–2.5% acrylamide gel. On all gels, from above downwards high, intermediate and low molecular weight multimer bands can be seen in normal, while for the II_A patients the high and intermediate molecular weight multimers are absent. With severe vWD patient (LW) no bands are seen. Qualitative differences, however, are seen on the different gels. Electrophoresis on 1.4% agarose gave the most poorly defined multimer pattern while that on 2% agarose was clearer with a wider spread and a further high molecular band (band 8) seen clearly. Definition and length of spread was best on 0.8% agarose–2.5% acrylamide gel.

The effect of changing electrophoretic conditions to obtain further resolution into the clearest multimeric pattern (Figs. 1, 2 and 3) is summarised in the Table. The clearest resolution was obtained with a starting current of 1.9 mA followed by 2.5 mA after sample migration from the well (see Methods) for 8 h and whereas bands 1–6 had appeared as single bands they could now be seen to be more complex with a dense central band flanked on either side by a fainter band. With this technique a final comparison was made between normal plasma and a type II_A variant. As well as the II_A patient lacking the high molecular weight multimers, there are qualitative differences in the triplet pattern of the lowest two triplets (band 1 and 2).

In the normal plasmas (see Figs. 3 and 4) the triplet pattern is of a central band with minor less densely stained bands on either side and designated as a and b. In the multimeric analysis of the type II_A patients studied here (Fig. 4) bands 1 to 4 are clearly seen. However, band 4 is weaker than band 4 in a normal plasma while band 3 is of similar intensity to the normal band 3 but the 3b band is much stronger in the II_A patients. Band 2 is dense and the a and b bands are denser than in the normal. Band 1 is also stronger in the II_A patients than in normals and the a and b bands are also more concentrated. Differences in the strength of the 1b band are seen between patients HW and CB (see Fig. 4).

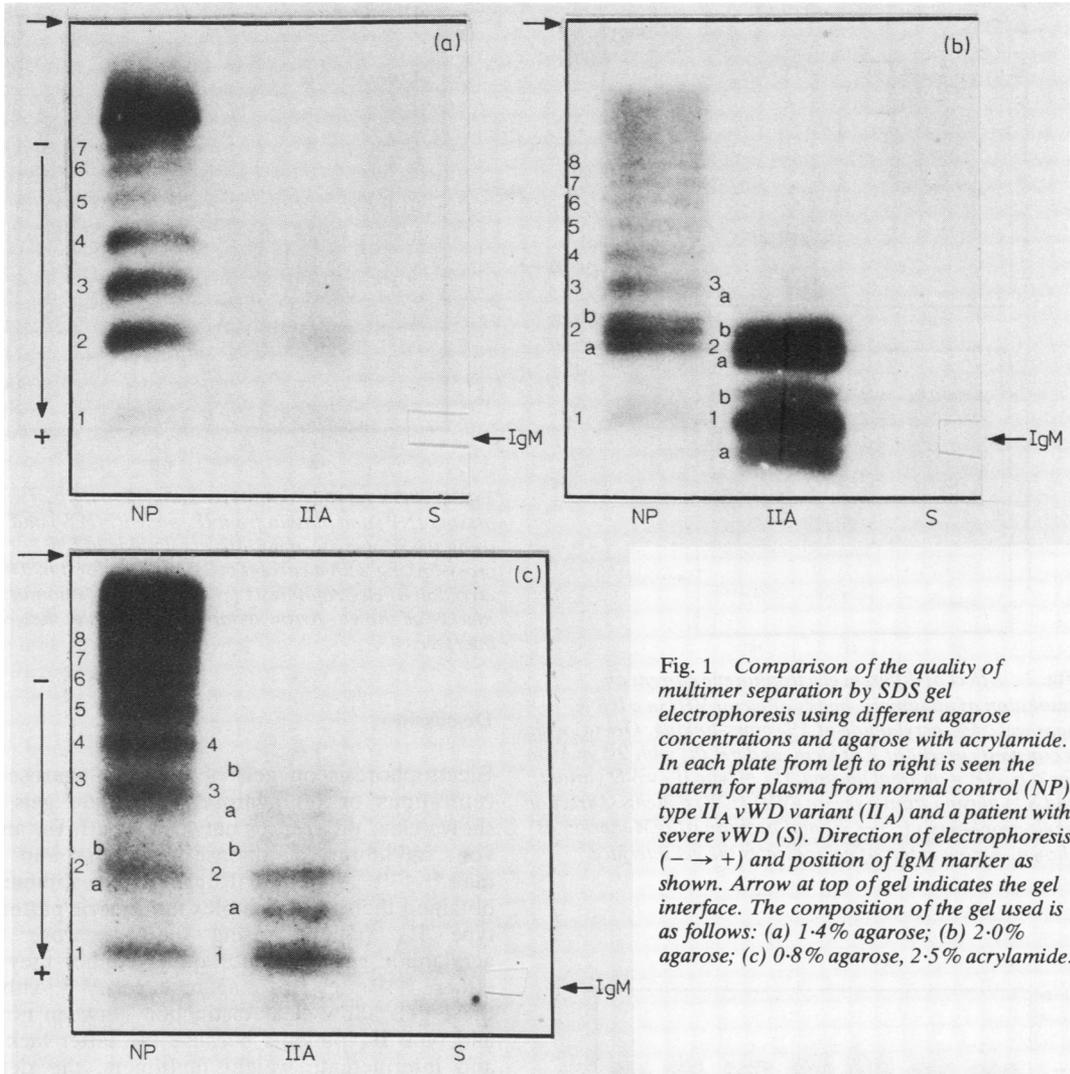


Fig. 1 Comparison of the quality of multimer separation by SDS gel electrophoresis using different agarose concentrations and agarose with acrylamide. In each plate from left to right is seen the pattern for plasma from normal control (NP), type II_A vWD variant (II_A) and a patient with severe vWD (S). Direction of electrophoresis (→) and position of IgM marker as shown. Arrow at top of gel indicates the gel interface. The composition of the gel used is as follows: (a) 1.4% agarose; (b) 2.0% agarose; (c) 0.8% agarose, 2.5% acrylamide.

Effect of different electrophoretic conditions on clarity of multimer separation

Current in mA/cm		Time (h) of electrophoresis	Quality of multimer separation with normal plasma
Prior to migration from well	After migration from well		
0.78	1	6	Poor separation
1.6	2	6	Resolution into six bands plus unresolved high molecular weight region. No triplet structure to bands (see Fig. 2 gel (a)).
1.9	2.5	6	Eight clear bands plus a smaller unresolved high molecular weight region. Bands 1 and 2 showing triplet appearance but not as three distinct bands (see Fig. 2 gel (b)).
1.9	2.5	8	Eight to twelve clear bands and small unresolved area of highest molecular weight multimers. Bands 1-6 showing triplet pattern. Doublet and single band anodal to band also seen clearly (see Fig. 3).

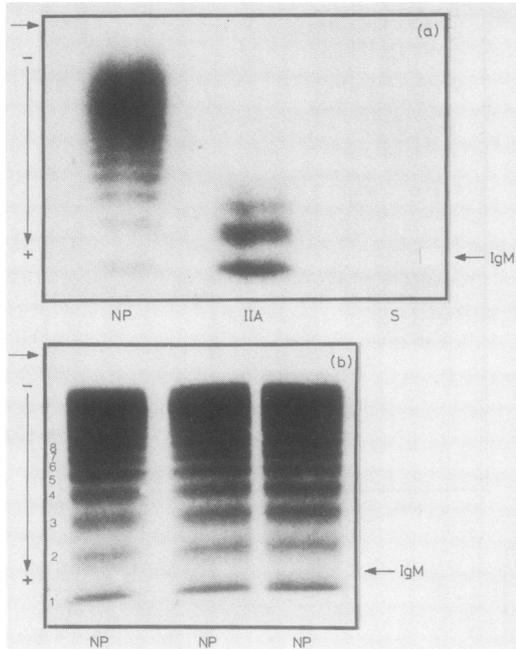


Fig. 2 Effect of different electrophoretic current on resolution of multimeric bands of factor VIII in a 0.8% agarose/2.5% acrylamide at 5% cross-linking. Gel (a) with a constant current of 2 mA/cm, and gel (b) with 2.5 mA/cm for 6 h. NP = normal plasma, II_A = type II_A vWD variant and S = plasma from a severe vWD (LW) patients. Direction of electrophoresis (- → +) and position of IgM marker as shown. Arrow at top of gel indicates the gel interface.

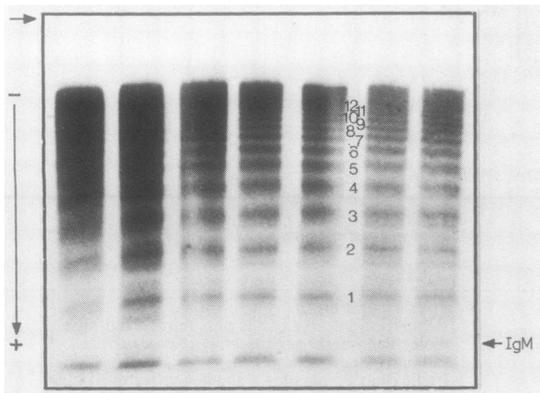


Fig. 3 SDS gel electrophoresis of normal plasma in 0.8% agarose/2.5% acrylamide at 5% cross-linking run for 8 h at 2.5 mA/cm. Direction of electrophoresis (- → +) and position of IgM marker as shown. Gel interface is indicated by arrow at top of gel.

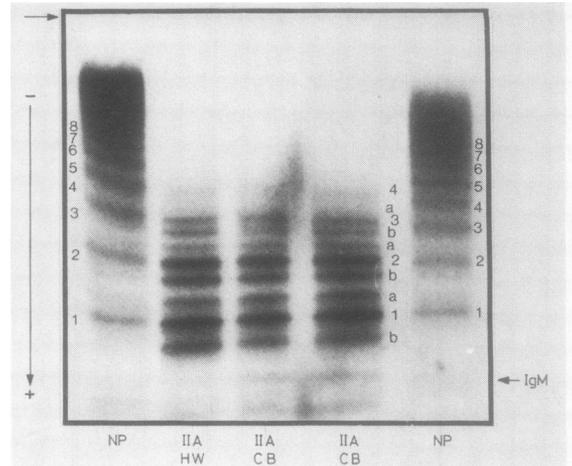


Fig. 4 SDS gel electrophoresis from left to right of normal plasma (NP) and plasma from II_A patients (HW) and (CB), using 0.8% agarose and 2.5% acrylamide at 5% cross-linking with a current at 2.5 mA/cm for 8 h. The direction of electrophoresis (- → +) and position of IgM marker as shown. Arrow at top of gel indicates the gel interface.

Discussion

Electrophoresis on gels of different agarose concentrations or on agarose acrylamide gels have shown clear differences between type I, II_A and II_B von Willebrand's disease patients and normals.¹¹⁻¹⁴⁻¹⁶ Although Ruggeri and Zimmerman obtained their most complex multimeric pattern for type II_A patients using 0.8% agarose 2.5% acrylamide gels,¹⁶ in subsequent studies they used either 1.4% or 1.6% agarose gels.¹⁸⁻¹⁹ Although these gels allow clear distinction between normals and type II_A patients because the latter lack high and intermediate weight multimers, the detailed complexity of the triplet configuration of the lower molecular weight multimers is not shown. In a recent report, a patient has been described who, in addition to lacking the high molecular weight multimers, has an absent triplet pattern and a repeating doublet pattern. None of the bands seen in these patients has the same mobility as bands seen in normals or previously described von Willebrand's disease patients. These patients have been classified as type II_C.²⁰

We have shown here how by optimising electrophoretic conditions it is possible to provide increasing resolution revealing a very complex multimeric pattern in normals and von Willebrand's disease variants. This better resolution shows that two different II_A variants (CB, HW) not only lack the

high and intermediate multimers but that the configuration of the triplet pattern of the lower two bands also differs from that seen in normals and in each other.

Application of this refined technique may, therefore, allow further reclassification of von Willebrand's disease patients and by use in family studies allow the identification of inherited asymptomatic multimer defects and how these may interact to produce symptomatic bleeding disorders.

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